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RESEARCH ARTICLE

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF *STREPTOMYCES* SPECIES SRDP-H03 ISOLATED FROM SOIL OF HOSUDI, KARNATAKA, INDIA

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ABSTRACT

In the present study, we report antibacterial and antioxidant activity of ethyl acetate extract of *Streptomyces* species SRDP-H03 isolated from rhizosphere soil of Hosudi, Karnataka, India. The isolate SRDP-H03 was assigned to the genus *Streptomyces* based on the cultural and microscopic characteristics. The ethyl acetate extract of the isolate SRDP-H03 showed marked inhibition of Gram positive bacteria than Gram negative bacteria. The extract was found to possess dose dependent DPPH free radical scavenging and Ferric reducing activity. UV spectral data of ethyl acetate extract showed strong absorption maxima (λmax) at 267 and 340nm. The isolate can be a potential candidate for the development of novel therapeutic agents active against pathogens and free radicals. Further studies on genomic characterization of isolate and isolation and structure determination of the bioactive compounds are under progress.

Key words: Hosudi, *Streptomyces*, Cross streak, Agar well diffusion, DPPH, Ferric reducing

INTRODUCTION

Microorganisms are attractive sources of bioactive compounds with pharmaceutical and agricultural importance. Actinomycetes, belonging to the order Actinomycetales, are Gram positive, filamentous eubacteria with high G+C content. They are involved in important processes in a wide range of habitats. They are responsible for the production of volatile compounds like geosmin responsible for characteristic earthy odor. They can degrade a variety of compounds such as lignocelluloses and many other polymers occurring in soil and litter, and a range of xenobiotic compounds. In addition, due of their metabolic diversity, actinomycetes are important source of lytic enzymes, antibiotics and other bioactive metabolites such as plant growth promoters, herbicides, insecticides, antitumor agents etc. These actinomycetes have been considered as biotechnologically and industrially valuable prokaryotes since they have produced a large number of compounds of pharmaceutical and agricultural importance [1-4].

Rhizosphere is a unique biological niche with a diverse microflora comprising bacteria, fungi, protozoa and algae. It is defined as the soil adjacent to and influenced by plant root exudates. The microbial community in rhizosphere is nutritionally favoured by input of organic materials derived from the plant roots and root exudates. Actinomycetes are important rhizosphere inhabitants of many plants, where they enhance plant growth and protect the plant roots against attack by phytopathogens. Most soil actinomycetes are saprophytic. Among the various actinomycetes genera, *Streptomyces* is the best recognized and well-studied genus of actinomycetes in terms of number and the bioactive metabolites being produced. The *Streptomyces* species are aerobic, spore formers with DNA rich in GC content (69-73 %). They form extensive branching substrate, aerial mycelia and widely distributed in soil. They have been recognized as prolific source of bioactive microbial metabolites as they produced 75% of biologically active compounds [2,3,5,6].

Hosudi (Latitude 13.9222; Longitude 75.6437) is a small village in Shivamogga (taluk), Shivamogga (district), Karnataka (State), India. It is located 10km away from the Shivamogga city. No microbial studies on the soil of this village have been carried out earlier. Hence, in the present study, we report antioxidant and antimicrobial activity of solvent extract of *Streptomyces* species SRDP-H03 isolated from a rhizosphere soil collected in Hosudi.

MATERIALS AND METHODS

Collection of soil sample

The soil sample was collected at Hosudi during the month of October 2012. The soil was collected in a sterile plastic bag from a depth of 15cm, immediately brought to the laboratory and dried at 40°C under aseptic conditions [7].

Isolation of actinomycetes

The dried soil sample was subjected to serial dilution (up to 10^{-5}) followed by plating on Starch casein agar (SCA) amended with antibiotic Fluconazole (to prevent fungal contamination). The plates were incubated aerobically at 30°C for 10 days. The colonies of actinomycetes were selected on the basis of typical morphology. The isolates were subcultured on sterile SCA slants and maintained in refrigerator [7].

Primary screening of the actinomycetes isolates

In order to screen antibacterial activity of actinomycetes isolates, we employed Cross streak method. Briefly, the actinomycetes isolates were streaked at the centre of SCA plates followed by incubation at 30°C for 5 days. After 5 days, the test bacteria were inoculated perpendicular to the

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growth of the actinomycetes isolates, incubated for 24 hours at 37°C and the extent of inhibition was observed. The absence of growth or a less dense growth of test bacteria near the actinomycetes isolates was considered positive for production and secretion of antibacterial metabolite by the isolates [8]. One isolate, SRDP-H03, showed marked inhibition of test bacteria and the isolate was selected for further studies.

Cultural characteristics of isolate SRDP-H03

The isolate SRDP-H03 was grown on various media namely SCA, Inorganic salt-starch agar (ISSA), Actinomycetes isolation agar (AIA) and Tryptone yeast extract agar (TYEA) and Yeast extract-Malt extract agar (YEMEA). The color of substrate and aerial mycelium and production of diffusible pigments were observed.

Microscopic characteristic of isolate SRDP-H03

The characteristic spore arrangement in the isolate SRDP-H03 was studied by employing cover slip method. Here, thin blocks of SCA were cut, placed on a sterile glass slide and the culture of SRDP-H03 was inoculated on the entire surface of the agar block. A sterile cover slip was placed on the inoculated surface; the slide was placed in a sterile moist chamber and incubated until good growth was obtained. The cover slip was then removed, placed on a drop of dilute crystal violet stain taken on a clean glass slide and observed under oil immersion objective in order to observe arrangement of spores [7].

Staining and biochemical characteristics of isolate SRDP-H03

The isolate SRDP-H03 was subjected for staining techniques namely Gram's and Acid-fast staining and biochemical tests namely starch hydrolysis, gelatin liquefaction, casein hydrolysis, catalse test, oxidase test, citrate test, cellulose hydrolysis, hydrogen sulfide (H_2S) production test and sugar fermentation tests [9,10].

Fermentation

Mass cultivation of the isolate SRDP-H03 was carried out in sterile Starch casein broth (SCB). The spore suspension of well sporulated culture of SRDP-H03 was inoculated into flasks containing sterile Starch casein broth and the flasks were incubated aerobically at 30°C for 10 days. The broth was filtered through sterile Whatman No. 1 filter paper [7].

Extraction of bioactive metabolite from culture filtrate

The culture filtrate was centrifuged and the supernatant was subjected solvent extraction using ethyl acetate. Equal volume (1:1) of supernatant and ethyl acetate were taken in a separation funnel and agitated for about 30 minutes. The solvent layer was separated and the supernatant was again extracted with ethyl acetate. The solvent layers were pooled and evaporated to dryness at 40°C [11]. The crude solvent extract thus obtained was screened for antibacterial and antioxidant activity.

UV absorption of ethyl acetate extract of SRDP-H03

The ethyl acetate extract was dissolved in ethyl acetate and subjected to UV absorption studies. The absorption spectrum of the extract was determined in the UV region (200-400nm) using a UV-visible spectrophotometer (Shimadzu UV 2554) [12].

Total phenolic content of ethyl acetate extract of isolate SRDP-H03

The total phenolic content of ethyl acetate extract was determined by employing Folin-Ciocalteu Reagent (FCR) method. Here, a dilute concentration of the extract (0.5 mL) was mixed with 0.5 ml of diluted Folin-Ciocalteu reagent (1:1) and 4 ml of sodium carbonate (1 M). The tubes were allowed to stand for 15 minutes and the absorbance was read colorimetrically at 765 nm. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/mL) and the total phenolic content of extract was estimated as µg Gallic acid equivalents (GAE)/mg of extract [13].

Antibacterial activity of ethyl acetate extract of isolate SRDP-H03

Agar well diffusion method was performed to screen antibacterial activity of ethyl acetate extract of SRDP-H03 against three Gram positive bacteria *viz*., *Staphylococcus aureus* NCIM- 2079, *Bacillus cereus* NCIM-2016 and *Bacillus subtilis* NCIM-2699 and five Gram negative bacteria *viz*., *Escherichia coli* NCIM-2685, *Vibrio cholerae* MTCC-3905, *Shigella flexneri* NCIM-4924, *Klebsiella pneumoniae* NCIM- 2957 and *Pseudomonas aeruginosa* NCIM-2242. The test bacteria were inoculated into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated for 24 hours at 37°C. Using sterile swabs, the broth cultures of test bacteria were swabbed on sterile Nutrient agar (HiMedia, Mumbai) plates followed by punching wells of 6mm diameter using sterile cork borer. 100µl of ethyl acetate extract (5mg/ml of 10% DMSO), standard (Streptomycin, 1mg/ml) and DMSO (10%) were transferred into labeled wells and the plates were incubated at 37°C for 24 hours. The plates were observed for zone of inhibition formed, if any, and measured using a ruler [7]. The experiment was carried in replicate and the average value was recorded.

Antioxidant activity of ethyl acetate extract of isolate SRDP-H03

DPPH free radical scavenging activity

DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay was performed to evaluate radical scavenging nature of extract of SRDP-H03 [14]. Briefly, 2ml of DPPH solution (0.002% in methanol) was mixed with 2ml of different concentrations (5-200µg/ml) of ethyl acetate extract and reference standard (ascorbic acid) in separate tubes. The tubes were incubated in dark at room temperature for 30 minutes and the optical density was measured at 517 nm using UV-Vis spectrophotometer. The absorbance of the DPPH control (without extract/standard) was noted. The scavenging activity was calculated using the formula:

Scavenging activity (%) = [(A – B) / A] x 100, where A is absorbance of DPPH control and B is absorbance of DPPH in the presence of extract/standard.

Ferric reducing activity

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In this assay, different concentrations (5-200µg/ml) of ethyl acetate extract of SRDP-H03 and ascorbic acid (reference standard) in 1ml of methanol were mixed separately with 2.5ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The tubes were incubated at 50°C for 20 minutes in water bath, cooled rapidly and mixed with 2.5ml of 10% trichloroacetic acid and 0.5 ml of 0.1% ferric chloride. After 10 minutes, the amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm. An increase in absorbance on increase in concentration indicates increased reducing power [14].

RESULTS

A total of 11 actinomycetes isolates (SRDP-H01 to SRDP-H11) were recovered from the soil of Hosudi on SCA. All the isolates were subjected for cross streak method in order to assess antagonistic property against a panel of 8 bacteria. Presence of clear zone or reduced growth of test bacteria near the growth of actinomycetes was considered as positive for antagonistic activity. All the isolates were potent enough to inhibit at least one of the test bacteria. The isolate SRDP-H03 showed prominent inhibitory activity against all test bacteria whereas least inhibitory effect was observed in case of isolate SRDP-H11 **(Table 1)**.

Table 1: Preliminary screening for antibacterial activity of actinomycetes isolates

Isolates	Extent of inhibition of test bacteria							
	E. coli	*S. flexneri*	*K. pneumoniae*	*B. cereus*	*V. cholerae*	*S. aureus*	*B. subtilis*	*P. aeruginosa*
SRDP-H01	+	++	++	+	+	++	+	+
SRDP-H02	-	++	++	++	+	+++	+	-
SRDP-H03	++	++	++	+++	++	+++	+++	++
SRDP-H04	+	+	+	++	-	++	+	-
SRDP-H05	-	+	+	+	-	++	+	+
SRDP-H06	+	+	-	++	-	+++	+	+
SRDP-H07	+	+	++	++	+	+	+	-
SRDP-H08	-	-	+	-	-	++	+	-
SRDP-H09	+	++	+	+	+	+	+	-
SRDP-H10	-	+	++	+	+	+	-	-
SRDP-H11	-	-	-	+	+	-	-	-

The isolate SRDP-H03 was Gram positive and non-acid fast. The spore arrangement was flexous type. The isolate showed positive result for amylase, cellulase, catalase, gelatinase and citrase production. Production of oxidase, caseinase and H_2S was not detected. The isolate fermented glucose and fructose with acid production and galactose and lactose with alkali production. In case of maltose, acid or alkali production was not observed. No gas production was observed in carbohydrate fermentation tests **(Table 2)**. Based on the cultural and microscopic characterisitics, the isolate SRDP-H03 was assigned to the genus *Streptomyces*.

The growth characteristics of the isolate SRDP-07 was studied on five media *viz.*, SCA, ISSA, AIA, TYEA and YEMEA. The growth was good on SCA, ISSA and TYEA whereas moderate growth was observed on AIA and YEMEA. The color of substrate and aerial mycelium varied in different media. The color of substrate mycelium was yellow, greenish yellow and cream whereas aerial mycelium showed variations in color namely grey, light grey and white. Diffusible pigments were not observed in any of these media **(Table 3; Figure 1)**.

Table 2: Microscopic, Staining and Biochemical characteristics of the isolate SRDP-H03

Test	Characteristic
Gram's staining	Gram positive
Acid fast staining	Non-acid fast
Spore arrangement	Flexous
Catalase test	+
Oxidase test	-
Starch hydrolysis	+
Cellulose hydrolysis	+
Gelatin liquefaction	+
Casein hydrolysis	-
Citrate test	+
H_2S production	-
Fermentation	Glucose- Acid Fructose- Acid Maltose- No acid/alkali Galactose- Alkali Lactose- Alkali

Table 3: Cultural characteristics of isolate SRDP-H03 on various media

Media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigment
SCA	Good	Yellow	Grey	-
ISSA	Good	Greenish yellow	Light grey	-
AIA	Moderate	Cream	Light grey	-
TYEA	Good	Yellow	Grey	-
YEMEA	Moderate	Yellow	White	-

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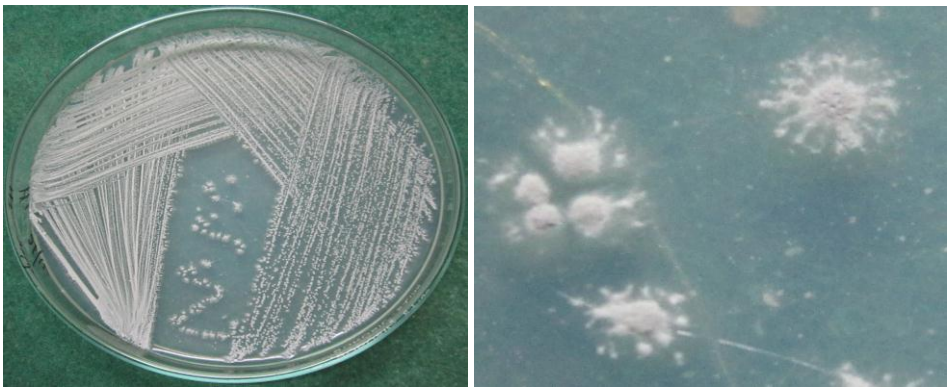

Figure 1: *Streptomyces* species SRDP-H03 on SCA

UV spectral data of ethyl acetate extract exhibited strong absorption maxima (λmax) at 267 and 340nm. Total phenolic content of ethyl acetate extract was found to be 8.75µg GAE/mg extract.

The efficacy of ethyl acetate extract of SRDP-H03 to inhibit bacteria was tested against a panel of 8 bacteria. It was observed that the inhibition of Gram positive bacteria by the extract was highest when compared to Gram negative bacteria. Among Gram positive and Gram negative bacteria, high susceptibility to extract was shown by *S. aureus* and *K. pneumoniae* respectively. However, the inhibitory effect of extract was lesser than that of standard antibiotic. DMSO (10%) did not show any inhibition of test bacteria **(Table 4)**.

Table 4: Antibacterial activity of ethyl acetate extract of isolate SRDP-H03

Test bacteria	Zone of inhibition in cm		
	Ethyl acetate extract	Streptomycin	DMSO
S. aureus	1.8	4.5	0.0
B. cereus	1.7	4.1	0.0
B. subtilis	1.7	3.8	0.0
K. pnuemoniae	1.4	3.4	0.0
P. aeruginosa	1.3	3.5	0.0
E. coli	1.3	2.9	0.0
V. cholerae	1.2	3.1	0.0
S. flexineri	1.2	3.6	0.0

Figure 2 shows the result of radical scavenging effect of ethyl acetate extract and ascorbic acid. Both extract and ascorbic acid exhibited dose dependent scavenging of DPPH radicals. Though the extract was able to scavenge DPPH* (free radical) and converting it into DPPHH, the scavenging effect of the extract was lesser than that of ascorbic acid. The radical scavenging effect of extract was >50% at concentration of 100µg/ml and higher.

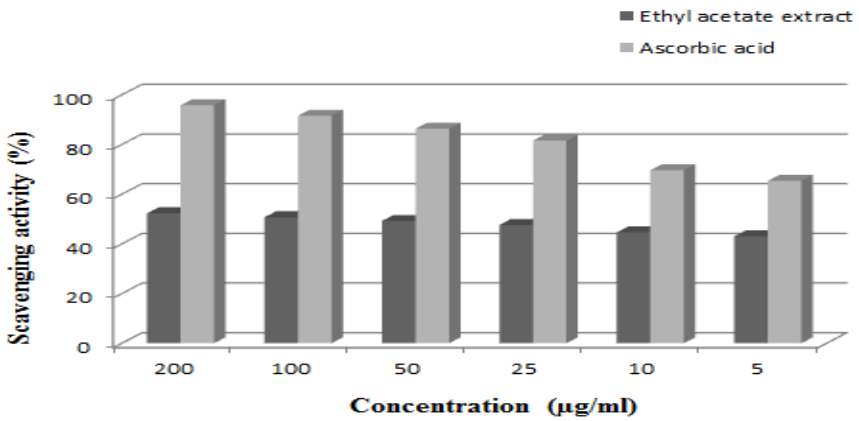

Figure 2: DPPH radical scavenging activity of ethyl acetate extract and ascorbic acid

The reducing power was determined by the reduction of Fe^{3+} to Fe^{2+} in the presence of different concentrations of ethyl acetate extract and ascorbic acid. The absorbance of reaction mixture at 700nm increased with the increase in concentration of extract indicating reducing potential of extract. However, the reducing potential of extract was lesser than that of reference standard **(Figure 3)**.

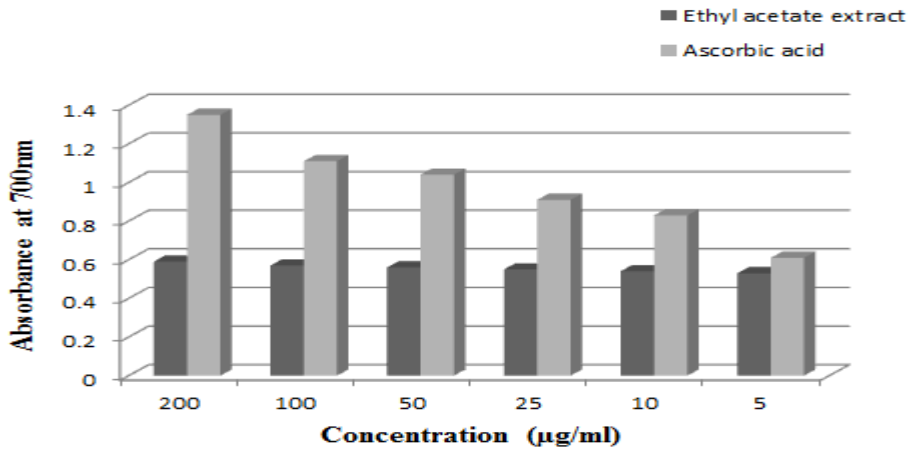

Figure 3: Ferric reducing activity of ethyl acetate extract and ascorbic acid

DISCUSSION

The plant root primarily determines the nature and abundance of the rhizosphere soil microflora, when conditions affect root growth or metabolism, it will be reflected in quantitative and qualitative changes in microbial populations of rhizosphere [15]. Rhizosphere, the soil present in vicinity to plant roots, is a unique ecological niche inhabited by diverse group of microflora such as bacteria, fungi, algae and protozoa. During growth, active roots are continuously exuding a range of organic compounds (i.e., mainly carbohydrates, carboxylic acids, and amino acids) directly into the rhizosphere. This nutrient input from plant roots significantly influence associated soil microbial communities. In the soil, microorganisms preferentially colonize the rhizosphere because root exudates are a major source of nutrients in soils, making the rhizosphere an area of intense activity. Conversely, microbial communities can affect rooting patterns, stimulate and promote plant root growth (e.g., release of hormones, neutralizing toxic substances, etc.), and influence the supply of available nutrients for plant uptake. Microbial turnover of rhizodeposits plays an important role in carbon flow through soils [6, 11, 16,17].

Actinomycetes are an important class of bacteria and constitute one of the important groups of the rhizosphere microflora. It has been shown that actinomycetes are quantitatively and qualitatively important in the rhizosphere, where they are known to influence plant growth and protect plant roots from pathogenic fungi. Members of *Streptomyces* are most abundant in soil and accounts for about 90% of actinomycetes isolated from soil [3,6,11,18]. In the present study, we have recovered 11 actinomycetes isolates from a rhizosphere soil collected at Hosudi. All the isolates were subjected to primary screening for antibacterial activity by cross streak method. This dual culture method is widely used to screen the ability of actinomycetes strains to produce antimicrobial metabolites [7,8,19,20]. Out of 11 isolates, 2 isolates have shown inhibition of all test bacteria. We have selected a potent isolate SRDP-H03 which inhibited all test bacteria to high extent.

The members of *Streptomyces* can be distinguished from other sporing actinomycetes based on morphology and hence morphology plays an important role in the characterization of *Streptomyces* species. The life cycle of Streptomycetes provides 3 distinct features for microscopic characterization namely vegetative mycelium, aerial mycelium baring chains of spores and the characteristic arrangement of spores and the spore ornamentation. The latter two features produce most diagnostic information [21,22]. Details on cultural and microscopic characteristics together with biochemical properties assisted the researchers to classify actinomycetes as members of the genus *Streptomyces*. Many studies have been carried out where the actinomycetes isolates were identified as species of *Streptomyces* based these properties or characteristics [7,14,20,23-27]. In the present study, the cultural and microscopic characteristics of the isolate SRDP-H03 were consistent with its classification as a member of the genus *Streptomyces*.

Antimicrobial agents play an indispensable role in decreasing morbidity and mortality associated with infectious diseases caused by bacteria, fungi, viruses and parasites. However, selective pressure exerted by the use of antimicrobial drug became the major driving force behind the emergence and spread of drug-resistance pathogens. In addition, resistance has been developed in pathogens after discovery of major class of antimicrobial drugs, varying in time from as short as 1 year in case of penicillin to >10 years in case of vancomycin [28]. This alarming situation necessitated search new bioactive compounds capable of acting against pathogens in particular drug resistant pathogens. It is well known that microorganisms, in particular bacteria and fungi are an unexhaustible source of natural compounds having several therapeutic applications. In the present study, it was found that Gram positive bacteria were susceptible to high extent than Gram negative bacteria. Similar findings were obtained in earlier studies of Kekuda *et al.* [7], Hassan *et al.* [29], Anansiriwattana *et al.* [30], Al-Hulu *et al.* [31], Valli *et al.* [32], Manasa *et al.* [33] and Gunda and Charya [34] which report high susceptibility of Gram positive bacteria than Gram negative bacteria. The low antibacterial activity of ethyl acetate extract against the gram negative bacteria could be ascribed to the presence of an outer membrane that possess hydrophilic polysaccharides chains and forms an additional barrier for the entry of extract as well as antibiotics into the cells [35,36].

DPPH is a stable, organic and nitrogen centered free radical having the absorption maximum band around 515-528nm (517nm) in alcoholic solution. It accepts an electron or hydrogen atom and becomes a stable diamagnetic molecule. Though a number of *in vitro* assays have been developed to evaluate radical scavenging activity of compounds, the model of scavenging of the stable DPPH radical is one of the widely used protocols. The effect of antioxidants on scavenging DPPH radical is due to their hydrogen donating ability. In this assay, the antioxidants reduce the purple colored DPPH radical to a yellow colored compound diphenylpicrylhydrazine, and the extent of reaction will depend on the hydrogen donating ability of the antioxidants [37,38,39]. In the present study, a decrease in the absorption of DPPH solution in the presence of various concentrations of ethyl acetate extract was measured at 517nm. It was observed that the radical scavenging activities of extract and ascorbic acid increased on increasing concentration. The scavenging effect of ethyl acetate extract of SRDP-H03 was much lesser when compared with ascorbic acid. Similar results were observed in earlier studies of Kekuda *et al.* [14], Manasa *et al.* [33], Priya *et al.* [40], Thenmozhi and Kannabiran [41] and Gautham *et al.* [42] where the extract showed low radical scavenging potential. Although the scavenging abilities of extract was lesser, it was evident that the extracts showed hydrogen donating ability and therefore the extract could serve as free radical scavengers, acting possibly as primary antioxidants [38].

The Fe^{+3} to Fe^{+2} transformation in the presence of varying concentrations of ethyl acetate extract of SRDP-H03 was investigated in order to assess the reducing power of the extract. In this assay, the presence of reductants (antioxidants) in the extract would result in the reduction of Fe^{+3} to Fe^{+2} by donating an electron. The amount of Fe^{+2}complex can be monitored by measuring the formation of Perl's Prussian blue at 700nm. Increasing absorbance at 700nm with increase in concentration of extract indicates an increase in reductive ability [38]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [43]. In the present study, we found that the reducing power of the extract and ascorbic acid increased with the increase of their concentrations. Though reducing power of extract was lesser than that of ascorbic acid, it is evident that the extract possesses reductive potential and could serve as electron donors, terminating the radical chain reactions [38]. Similar observations have been made in previous studies of Kekuda *et al.* [14], Manasa *et al.* [33], Thenmozhi and Kannabiran [41] and Gautham *et al.* [42] where the extracts have displayed weaker reducing potential.

CONCLUSION

The study was successful in the isolation of antagonistic actinomycetes from a soil sample collected at Hosudi, Karnataka, India. The isolate SRDP-H03 was identified as a species of the genus *Streptomyces* based on cultural and microscopic characteristics. The ethyl acetate extract of the isolate SRDP-H03 showed antibacterial and antioxidant efficacy. The extract was found to contain bioactive principles which are to be separated and subjected for activity determinations. The isolate SRDP-H03 can be a potential candidate for the development of therapeutic agents. Further studies on characterization of the isolate and the purified bioactive components in the solvent extract are under progress.

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